

Role of sodium nitrate concentration in BG-11 medium used for autotrophic growth of *Anabaena vaginicola* cyanobacterium

B. Baniasadi, F. Najafi, F. Vahabzadeh*

*Department of Chemical Engineering, Amirkabir University of Technology (Tehran Polytechnic), Tehran, Iran
far@aut.ac.ir*

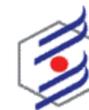
Abstract

Because of its multiple applications in biotechnology, in recent years, there has been a considerable focus on cyanobacteria as a nitrogen fixer prokaryote capable of carrying out photosynthesis. Due to the high lipid content present in their thylakoid membranes, cyanobacteria have a considerable potential to be used as a source of raw material in third generation biofuels. The present study was undertaken to characterize the autotrophic growth of *Anabaena vaginicola* (*A. vaginicola*) as the cyanobacterium under different concentrations of sodium nitrate. NaNO_3 is the main source of nitrogen in BG-11 medium for autotrophic growth of cyanobacteria. By changing the concentration of NaNO_3 in the formulation of BG-11 medium, it was found that growth rate constants of the cultures with different initial concentrations of NaNO_3 differed significantly from each other, suggesting that the initial NaNO_3 concentrations in BG-11 medium can have considerable effects on the growth of *A. vaginicola*. Absorbance readings for *A. vaginicola* culture grown in BG-11 media containing 1.875 and 2.25 g/L NaNO_3 were highest among seven groups of cultures and expressed highest specific growth rate (0.386 and 0.408 day^{-1} , respectively). BG-11 medium prepared with NaNO_3 at the lower level than the original amount of this inorganic salt (25% of 1.5 g/L) was not able to fully support the growth of *A. vaginicola* culture and the longer exponential phase of growth was with the lower rate constant (0.191 day^{-1}). This growth rate in comparison with the growth in the nitrate-free BG-11 medium (BG-11₀) was also lower.

Keywords: Cyanobacteria, *Anabaena vaginicola*, autotrophic growth, nitrogen fixation, BG-11 medium, sodium nitrate

Introduction

Increasing energy demand, environmental concerns, and depletion of fossil fuel sources are subjects that have generated great interest for searching reasonable alternative energy sources such as biofuels production from biomass. For instance, in case of biodiesel production, a wide range of feedstocks can be used and among them, photosynthetic prokaryotes have gained a special position. Association between growth and lipid production in microalgae and cyanobacteria has been the topic of many studies in the last decade. High growth rate of cyanobacteria as photosynthetic microorganisms has found to be related to accumulation of lipids [1]. Therefore, carbon dioxide absorption by these microbes, with smaller land requirement (compared to other seed oils as food crops) have made microorganisms a suitable alternative for biofuel production [2]. Because these organisms can fix carbon photosynthetically, they don't need any fermentable sugars as a carbon feedstock [3]. While



eukaryotic microalgae have the ability to accumulate large amounts of lipid under stress conditions with slow growth, cyanobacteria, the only oxygenic photosynthetic prokaryotes, can produce lipid with a fast growth due to their lipid accumulation in thylakoid membranes, their high levels of photosynthesis, and rapid growth rate [4]. Furthermore, cyanobacteria, which secretes fatty acids and other carbon-based products into their surrounding environment, is a tractable and naturally transformable host that can be genetically manipulated more easily than eukaryotic microalgae [1,3].

Growth condition and nutrient composition in culture medium of microalgae and cyanobacteria can have considerable impacts on their lipid content. When microorganisms such as cyanobacteria grow in the media with a rich carbon source and a limiting amount of nitrogen, they quickly use up the supply of nitrogen, but assimilation of the carbon source continue. As a result, lipid accumulation occurs [4]. However, nitrogen starvation does not always promote lipid induction. It can enhance the lipid production in many microalgae and cyanobacteria such as *Chlorella* sp., *Nannochloropsis* sp., *Neochloris oleoabundans*, *Botryococcus braunii*, *Microcystis panniformis* and *M. novacekii* [5,6].

Many types of cyanobacteria can fix atmospheric nitrogen through a highly endergonic reaction [7] (see eq.1)



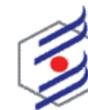
Anabaena sp., a diazotrophic cyanobacterium, is one of the few bacteria capable of producing specialized nitrogen-fixing heterocysts and fixing atmospheric dinitrogen into ammonia form of nitrogen with the help of oxygen-labile nitrogenase complex present in heterocysts cells [6,8]. All cyanobacteria can utilize different types of combined nitrogen as in nitrate, nitrite, and ammonium as the sole nitrogen source, while in absence of combined nitrogen, heterocystous cyanobacteria will differentiate vegetative cells into heterocysts at semiregular intervals between strings of vegetative cells [6,8]. Nitrogen fixation is catalyzed by oxygen-sensitive nitrogenase enzyme complex, that catalyzes the reduction of atmospheric nitrogen to ammonia [8]. Strength of nitrogen fixation process is decreased or even being inhibited in the presence of external combined nitrogen sources such as NO_3^- or NH_4^+ compounds [7,9]. Meeks et al. [10] found that nitrate have a strong inhibitory effect on the formation of specialized heterocyst cells in *Anabaena* sp. strain 7120.

The concentration of sodium nitrate as an exogenous nitrogen source in BG-11 culture medium is a key factor in growth and lipid production of microalgae and cyanobacteria. Therefore, nitrogen manipulation is considered a cost-effective strategy that can result in high lipid production in these microorganisms [6]. Despite the extensive research on the utilization of microalgae as an alternative source of energy, there are relatively fewer studies on the potential of using cyanobacterial biomass as a rich source of biofuels [11]. Hence, characterization of growth of cyanobacteria under different nitrate stress conditions can be useful in assessing the lipid productivity of cyanobacteria.

The aim of the present work is to study the effect of sodium nitrate concentration used in BG-11 medium on autotrophic growth of *A. vaginicola* cyanobacterium. The optical density, as a measure of biomass, and specific growth rate constants were determined in this regard.

Experimental Method

A pure culture of *Anabaena vaginicola* cyanobacterium was obtained through a previous work from this laboratory as reported in [12]. Cultivation of the culture was initially done using



standard cylinder (1L bottles), with a sterilized BG-11 medium at $28^{\circ} \pm 1^{\circ}$ C. BG-11 media consisted of (g/L): 1.5 NaNO₃, 0.036 CaCl₂·2H₂O, 0.012 FeNH₄-Citrate, 0.001 Na₂-EDTA, 0.04 K₂HPO₄, 0.075 MgSO₄·7H₂O, 0.02 Na₂CO₃, and 1 ml Haagland's micronutrients per liter of culture medium [8]. The cyanobacteria cells were cultured in 250 ml bottles containing 200 ml modified BG-11 fermentation medium with exposure to different concentration of NaNO₃ (0, 0.375, 0.75, 1.125, 1.5, 1.875 and 2.25 g/L) that are associated with 0, 25, 50, 75, 100, 125 and 150% of NaNO₃ concentration used in sBG-11 media, respectively. Other components of the BG-11 growth medium were used at the defined concentration according to the literature [8]. Autotrophic growth was followed using four 40-watt daylight fluorescent lights (continuous illumination of 80 μmol.photons.m⁻².s⁻¹). The top of the culture bottles was covered using sterilized cotton gauze balls. The cultures aeration was done using aquarium pump where the tubing was placed at the bottom of the vessel and 0.22 μm filtered air was injected into the cultures. In this manner of aeration, the cyanobacterial biomass did not settle in the vessel, and furthermore, the cultures had access to atmospheric CO₂. Trend of *A. vaginicola* growth was monitored daily and the absorbance was measured with spectroscopy technique using visible spectrophotometer (JASCO V-550 UV/VIS spectrophotometer, Japan) at a 750 nm wavelength. The specific growth rate constant (μ) of each cultivation sample was calculated using eq.2.

$$\mu = (\ln(OD_1) - \ln(OD_0)) / (t_1 - t_0) \quad \text{eq.2}$$

where OD₀ and OD₁ are optical density values (at 750 nm) at the beginning (t₀) and the end (t₁) of exponential phase, respectively.

Data analysis was performed using the PASW (SPSS) statistics 22 software (SPSS Inc. Chicago, IL, USA) where one-way analysis of variance (ANOVA) was used for comparisons between different cultural conditions in terms of sodium nitrate concentration and significance of F-test was further analyzed using Duncan method.

Results and Discussion

Importance of nutritional nitrogen source for growth of cyanobacteria is well-recognized. The present study was an attempt directed to monitor the trend of nitrogen utilization by *A. vaginicola* as an index for the microbe growth (Gr). In particular, the change of concentration of NaNO₃, which is the main ingredient in BG-11 formulation, was of the interest. The growth curves of *A. vaginicola* were obtained by recording absorbance values from exponential up to the stationary phase. Fig. 1 shows the time dependency of absorbance readings used as an estimate of the culture growth, where the highest readings obtained in the cultures with the highest initial NaNO₃ concentrations of 1.875 and 2.25 g/L. The trend of absorbance readings at the end of the exponential phase of the cultures (as a biomass production indicator) is as follows:

$$\text{Gr}_{0.375 \text{ g/L}} < \text{Gr}_{0 \text{ g/L}} < \text{Gr}_{0.75 \text{ g/L}} < \text{Gr}_{1.125 \text{ g/L}} < \text{Gr}_{1.5 \text{ g/L}} < \text{Gr}_{1.875 \text{ g/L}} < \text{Gr}_{2.25 \text{ g/L}}$$

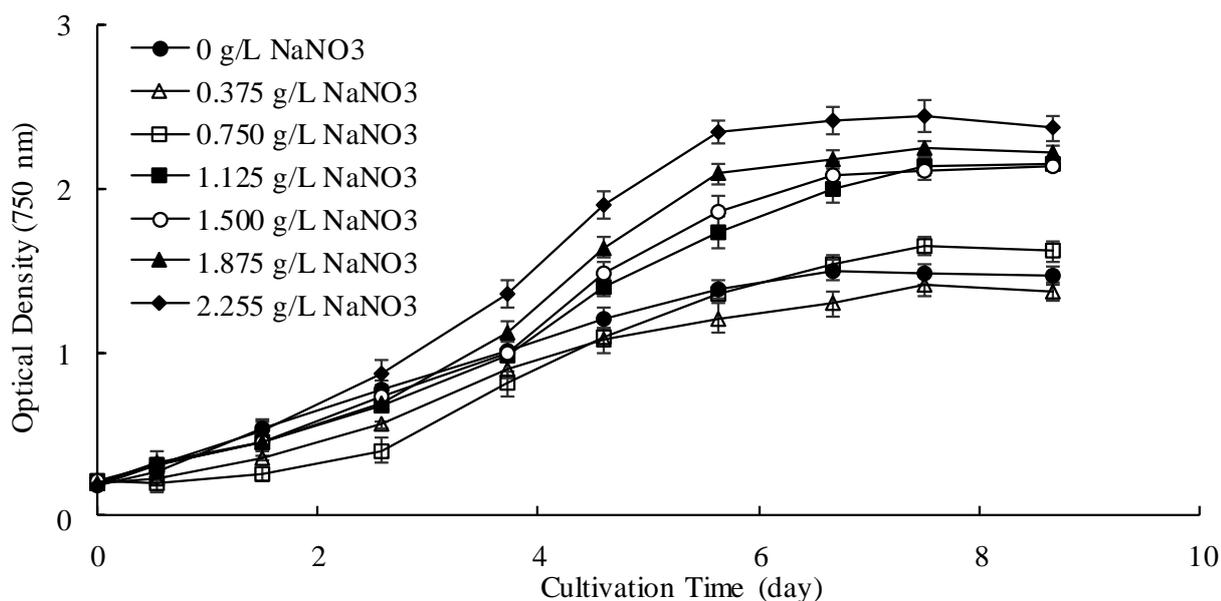
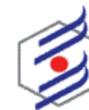


Fig. 1. Growth curves of *A. vaginicola* cultivated in BG-11 and modified BG-11 media with different concentrations of NaNO₃ (average of two replicates from independent cultures).

At NaNO₃ concentrations lower than 1.5 g/L, nitrogen starvation appeared, and the growth rate of *A. vaginicola* decreased. Also, the pigment loss (green color → yellow color) in *A. vaginicola* growth on BG-11 media having NaNO₃ at 0.375 and 0.750 g/L was considerable. Fig. 2 shows the values of specific growth rate constant (μ) which is the expression used to describe the typical growth curve. μ values confirm the growth trend seen in Fig. 1. Due to the decrease in the concentrations of nutrients in culture medium during growth in batch cultures, μ is not constant [13]. Nevertheless, in this work, this effect was assumed to be negligible and the values reported in the Fig. 2 were calculated as stated in the section 2. The concentration of NaNO₃ used in preparation of the modified BG-11 media (i.e. independent variable) significantly affected the values of μ obtained experimentally, and the findings were reported according to the results of one-way ANOVA test. The findings are in agreement with previous studies on *Microcystis panniformis*, *M. novacekii* [5] and *M. aeruginosa* [14] cyanobacteria. As shown in Fig. 1, cultures with different initial NaNO₃ concentration did not reach their stationary phase at the same time. This highlights the influence of initial NaNO₃ concentration in the BG-11 medium on the metabolism and growth of cyanobacteria. Presence of NaNO₃ at higher concentration in BG-11 medium preparation (1.875 and 2.25 g/L), resulted the stationary phase being reached after 5 days compared to 7 days typically observed in the growth curve of *A. vaginicola* grown in sBG-11 medium. With regards to the values of the growth rate constants and half-life time, the following comparison shows the performance of *A. vaginicola* grown in aforementioned cultures in formation of the biomass led to the higher amount compared to cultures with 25, 50 and 75% of NaNO₃ concentration used in sBG-11 media (at the 95% confidence level).

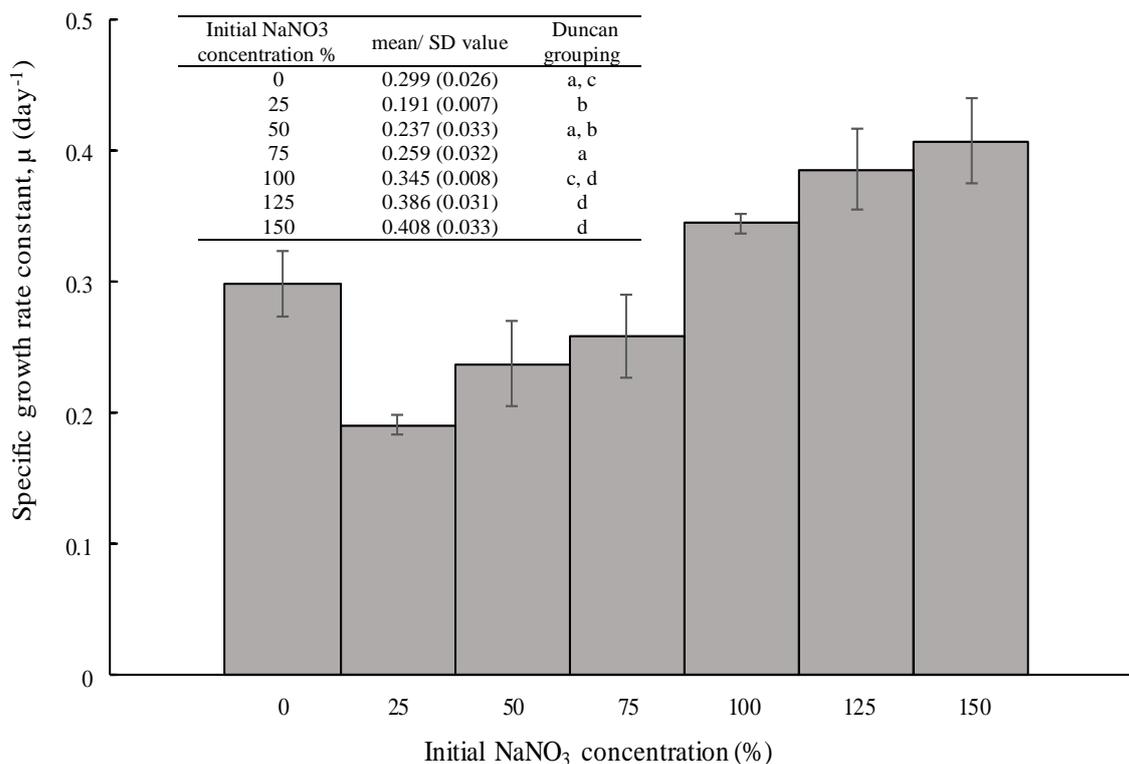
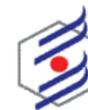
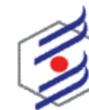


Fig. 2. Specific growth rate constants for growth of *A. vaginicola* culture in BG-11 growth medium modified on basis of NaNO₃ concentration. Inset: Duncan test used for comparison between means of the different groups (after applying ANOVA test). The values that do not share the common superscript are significantly different.

The results of the Duncan test show that for the cultures with NaNO₃ concentration of 0.375 g/L, the growth rate constant was considerably lower compared to *A. vaginicola* grown in BG-11₀. Also, the growth was delayed and less biomass was produced. This can be attributed to the fact that the presence of an alternative nitrogen source such as nitrate in growth culture system negatively affects the growth pattern of cyanobacteria. Moreover, low expression of nitrogenase activity has been found to be related to these observations most generally reported for heterocystous types of cyanobacteria.

Conclusions

In this work, autotrophic growth of *A. vaginicola* was characterized in different BG-11 culture media prepared with different initial NaNO₃ concentrations. μ values confirm the growth trends seen in Fig. 1. The cultures grown in BG-11 having 1.875 and 2.25 g/L NaNO₃ experienced shorter time to pass the exponential phase of growth. Moreover, biomass content and μ values of the test cyanobacteria grown in the aforementioned cultures were considerably higher compared to other cultures. More effort appears to be needed in order to decrease sodium nitrate level in BG-11 medium used for autotrophic growth of cyanobacteria. A reasonable approach to manage cost of biodiesel production is to use extracted lipids from cyanobacteria species and decreasing NaNO₃ content of BG-11 medium.



Acknowledgements

The present study was in continuation of S. Rezasoltani work [12], and the authors appreciate her assistance.

References

- [1] Lynch, F., Santana-Sánchez, A., Jämsä, M., Sivonen, K., Aro, E.M. and Allahverdiyeva, Y. , " Screening native isolates of cyanobacteria and a green alga for integrated wastewater treatment, biomass accumulation and neutral lipid production", *Algal Res.*, 11, 411–420 (2015).
- [2] Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M. and Chen, Y. , " Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance", *Bioresour. Technol.*, 102, 159–165 (2011).
- [3] Nozzi, N.E., Oliver, J.W.K. and Atsumi, S. , " Cyanobacteria as a platform for biofuel production", *Front. Bioeng. Biotechnol.*, 1, (2013).
- [4] Ratledge, C. , " Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production", *Biochimie*, 86, 807–815 (2004).
- [5] Cordeiro, R.S., Vaz, I.C.D., Magalhães, S.M.S. A and Barbosa, F.A.R. , " Effects of nutritional conditions on lipid production by cyanobacteria", *An Acad. Bras. Cienc.*, 89, 2021–2031 (2017).
- [6] Kumar, R., Biswas, K., Singh, P.K., Elumalai, S., Shukla, P. and Pabbi, S. , "Lipid production and molecular dynamics simulation for regulation of accD gene in cyanobacteria under different N and P regimes", *Biotechnol. Biofuels*, 10, 94 (2017).
- [7] Pinzon, N.M. and Ju, L.K. , " Modeling Culture Profiles of the Heterocystous N₂-Fixing Cyanobacterium *Anabaena flos-aquae*", *Biotechnol. Prog.*, 22, 1532-1540 (2006).
- [8] Videau, P., and Cozy, L.M. , " *Anabaena* sp. strain PCC 7120: Laboratory maintenance, cultivation, and heterocyst induction", *Curr. Protoc. Microbiol.*, 52 (1), e71 (2018).
- [9] Cheng, J., Hipkin C.R. and Gallon, J.R., "Effects of Inorganic Nitrogen Compounds on the Activity and Synthesis of Nitrogenase in *Gloeothoece (Nageli) sp. ATCC 27152*". *New Phytol.*, 141, 61-70 (1999).
- [10] Meeks, J.C., Wycoff, K.L., Chapman, J.S. and Enderlin, C.S. , " Regulation of Expression of Nitrate and Dinitrogen Assimilation by *Anabaena* Species", *Appl Environ. Microbiol.*, 45 (4), 1351–1359 (1983).
- [11] Karatay, S. E., Donmez, G. " Microbial oil production from thermophile cyanobacteria for biodiesel production", *Appl. Energy.*, 88, 3632-3635 (2011).
- [12] Rezasoltani S., Vahabzadeh, F., Shariatmadari Z. and Ghanati, F. " Cyanobacterial extract as a source of nutrients for mixotrophic growth of *Chlorella vulgaris* and *Nannochloropsis oculata*", *Algal Res.*, 39, 101480 (2019).
- [13] Ernst, A., Deicher, M. Herman, P.M.J. and Wollenzien, U.I.A. , " Nitrate and Phosphate Affect Cultivability of Cyanobacteria from Environments with Low Nutrient Levels", *Appl. Environ. Microbiol.*, 71 (6), 3379–3383 (2005).
- [14] Kim, H., Jo, B.Y. and Kim, H.S. , " Effect of different concentrations and ratios of ammonium, nitrate, and phosphate on growth of the blue-green alga (cyanobacterium) *Microcystis aeruginosa* isolated from the Nakdong River, Korea", *Algae*, 32 (4), 275-284 (2017).